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The Intracellular Pathogen *Rhodococcus equi* Produces a Catecholate Siderophore Required for Saprophytic Growth▽

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Little is known about the iron acquisition systems of the soilborne facultative intracellular pathogen *Rhodococcus equi*. We previously reported that expression of *iupABC*, encoding a putative siderophore ABC transporter system, is iron regulated and required for growth at low iron concentrations. Here we show that disruption of *iupA* leads to the concomitant accumulation of catecholates and a chromophore with absorption maxima at 341 and 528 nm during growth under iron-replete conditions. In contrast, the wild-type strain produces these compounds only in iron-depleted medium. Disruption of *iupU* and *iupS*, encoding nonribosomal peptide synthetases, prevented growth of the corresponding *R. equi* SID1 and SID3 mutants at low iron concentrations. However, only *R. equi* SID3 did not produce the chromophore produced by the wild-type strain during growth at low iron concentrations. The phenotype of *R. equi* SID3, but not that of *R. equi* SID1, could be rescued by coculture with the wild type, allowing growth at low iron concentrations. This strongly suggests that the product of the *iupS* gene is responsible for the synthesis of a diffusible compound required for growth at low iron concentrations. Transcription of *iupU* was constitutive, but that of *iupS* was iron regulated, with an induction of 3 orders of magnitude during growth in iron-depleted compared to iron-replete medium. Neither mutant was attenuated in vivo in a mouse infection model, indicating that the *iupU*- and *iupS*-encoded iron acquisition systems are primarily involved in iron uptake during saprophytic life.

The actinomycete *Rhodococcus equi* is a facultative intracellular pathogen that infects alveolar macrophages of young foals, causing pyogranulomatous lung lesions. In addition, *R. equi* is an opportunistic pathogen of immunocompromised humans, in particular of individuals diagnosed with AIDS, and sporadically infects other animals (27, 30). *R. equi* strains isolated from foals invariably harbor an 80- to 90-kb plasmid carrying a family of seven virulence-associated protein-encoding *vap* genes and two pseudo-*vap* genes within a 27-kb pathogenicity island (5, 33, 36, 40). The virulence plasmid is required for proliferation and cytoxicity of *R. equi* in macrophages. However, to date only VapA has been shown to be essential, although not sufficient, for virulence (18, 24). In addition to having this pathogenic lifestyle, *R. equi* is also a versatile saprophytic bacterium, capable of rapid growth in soil and manure (17).

Iron is essential for most forms of life, due to its involvement in many major biological processes as a biocatalyst and electron carrier. Although Fe^{2+} is soluble (0.1 M at pH 7), in the presence of oxygen Fe^{2+} is readily oxidized to the highly insoluble Fe^{3+}, (10^{-18} M at pH 7) (1). Iron is therefore growth limiting in most environments. To satisfy their iron requirements, many bacteria secrete low-molecular-weight iron-chelating compounds, generically known as siderophores, characterized by an extremely high affinity for Fe^{3+} (46). The three main types of siderophores contain catecholates, hydroxamates, or carboxylates as iron-coordinating functionalities; however, mixed siderophores and siderophores containing other functional groups such as diphenolates, imidazoles, and thiazolines have also been found (8). Following chelation of Fe^{3+} in the medium, siderophores are taken up by their cognate ABC transport systems, and Fe^{3+} release subsequently occurs either by reduction of Fe^{3+} to Fe^{2+} or by hydrolysis of the siderophore (22, 23, 28).

Pathogenic bacteria such as *R. equi* face a greater challenge because the iron concentration in the host's fluids and tissues is further lowered through binding to proteins such as hemoglobin, transferrin, lactoferrin, and ferritin (46). *R. equi* can acquire iron from holo-transferrin, iron-saturated lactoferrin, hemin, and hemoglobin, albeit with low efficiency (19, 29). To date, siderophores have not been detected in *R. equi* (12, 16). However, a mutant unable to grow at low iron concentrations contained an insertion in the *iupABC* operon encoding an ABC transport system similar to siderophore transporters, suggesting that *R. equi* produces at least one siderophore (29). The only rhodococcal siderophores characterized to date are heterobactins and rhodobactin from the nonpathogenic *Rhodococcus erythropolis* and *Rhodococcus rhodochrous*, respectively, containing catecholate and hydroxamate functionalities as iron-coordinating groups (6, 10). However, the genes encoding the enzymatic machinery for their biosynthesis remain unknown.

This paper describes the presence of an *R. equi* siderophore containing catecholate moieties, which is likely to be transported by the previously identified IupABC transport system (29). Knockout mutagenesis allowed the identification of a gene cluster encoding the nonribosomal peptide synthetase (NRPS) required for its biosynthesis. Virulence assays showed

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype, characteristics, or sequence	Source or reference
Strains		
E. coli DH5α	Cloning host strain; *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
R. equi strains		
ATCC 33701	Virulent strain, 81-kb virulence plasmid p33701	American Type Culture Collection
ATCC 33701 P^-	Avirulent strain, virulence plasmid cured	41
α5	*iupA*::(EZ::TN Kan2), derivative of ATCC 33701	29
SID1	*iupU*::pSID1K, derivative of ATCC 33701	This study
SID3	*iupS*::pSID3K, derivative of ATCC 33701	This study
Plasmids		
pAm1	Ap^r, *oriV*(ColE1), 2,427 bp	25
pSID1K	Suicide plasmid containing *sid1* PCR product cloned into the NcoI site of pAm1, 3,238 bp	This study
pSID3K	Suicide plasmid containing *sid3* PCR product cloned into the NcoI site of pAm1, 3,326 bp	This study
Oligonucleotides		
ApraF	5′-ACCGACTGGACCTTCCTTCT	This study
ApraR	5′-TCGGTCAGCTTCTCAACCTT	This study
Primers for cloning[a]		
SID1-KF	5′-CAATCT**ACATGT**GATAGCACGTACTGGTGATCGTC	This study
SID1-KR	5′-CATGCT**ACATGT**TCGGTGAGAGTGAGTTGCAG	This study
SID3-KF	5′-TCTGG**ACATGT**GATAGGACATCGTGGTCGGCACTG	This study
SID3-KR	5′-GATGCA**ACATGT**ACTCGTCGAGCACCAGGAC	This study
External primers[b]		
SID1-1032EF	5′-GGTCCAGGTGATCGTTCC	This study
SID1-1032ER	5′-AACAGGTCGGTCGCGTAG	This study
SID3-1060EF	5′-ATGTTCATGCTCTCGCACAC	This study
SID3-1060ER	5′-ATCCGGAGGTGTAGATGACG	This study
Primers for real-time PCR[c]		
SID1-220F	5′-CCTCCCTATCTCGTGGAACA	This study
SID1-220R	5′-CCTCCCCGTACACGTACAAC	This study
SID3-205F	5′-TTGGGTATTCACCACCTCGT	This study
SID3-205R	5′-CGAGGTAGTCGGTCCAGAAC	This study
SID4-193F	5′-GGGGCTGCACTACCTCAGTA	This study
SID4-193R	5′-CTTACGCGGACGGAAACAC	This study
16SrRNA200F	5′-ACGAAGCGAGAGTGACGGTA	29
16SrRNA200R	5′-ACTCAAGTCTGCCCGTATCG	29

[a] Primers used to produce PCR fragments to clone into the NcoI site of pAm1. NcoI-compatible PciI sites were added (bold). Underlining indicates ●●●●.
[b] External primers were used in combination with ApraF and ApraR primers to check the knockout.
[c] Primers used for real-time PCR.

that this siderophore is required for the saprophytic growth of *R. equi*. The resulting mutant was not attenuated for virulence, suggesting that *R. equi* must possess at least one additional system to acquire iron in the host. Since this mutant was unable to grow under low-iron conditions, this siderophore biosynthesis system seems to be required primarily for saprophytic growth.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* and *R. equi* were grown on Luria-Bertani (LB) medium (37) or on minimal medium supplemented with 20 mM lactate (LMM) at 37°C as previously described (20). To control the availability of iron in liquid media, LMM was extracted in batch with Chelex-100 ion exchanger resin following the manufacturer's recommendations (Bio-Rad) and supplemented with 30 μM thiamine and Vishniac-Santer trace metal solution (43) for iron-replete conditions (LMM) or with the same solution but lacking $FeSO_4$ for low-iron conditions (LMM-Fe). All cultures were performed in polycarbonate flasks thoroughly rinsed with Chelex-100-treated water. The iron content of media was determined by atomic absorption spectroscopy in a SpectrAA-10 instrument (Varian Inc.) using an Fe^{3+} SpectroSol solution as a standard. To study the secretion of iron-chelating compounds and examine the effect of iron availability on transcription of selected genes, *R. equi* was inoculated at an optical density at 600 nm (OD_{600}) of 0.05 and pregrown on LMM until the culture reached an OD_{600} of 0.6. At this point, cells were harvested, washed twice with Chelex-100-treated phosphate-buffered saline, and reinoculated in a fresh 50 ml of LMM or LMM-Fe at an OD_{600} of 0.05. The sensitivity of *R. equi* mutants to low-iron conditions was assessed by growth inhibition assays on LB agar containing 2,2′-dipyridyl. Growth inhibition was recorded as the

inability of the bacteria to develop isolated colonies after 3 days of incubation at 37°C. When appropriate, the following supplements were added: ampicillin, 50 μg ml^{-1}; apramycin, 30 μg ml^{-1} (*E. coli*) or 80 μg ml^{-1} (*R. equi*); kanamycin, 50 μg ml^{-1} (*E. coli*) or 200 μg ml^{-1} (*R. equi*); X-Gal (5-chromo-4-chloro-3-indolyl-β-D-galactopyranoside), 20 μg ml^{-1}; and 0.1 M isopropyl-β-D-thiogalactoside (IPTG). Agar was added for solid media (1.5% [wt/vol]).

DNA manipulations. Plasmid DNA was isolated with the alkaline lysis method (3) or by using the Wizard Plus SV miniprep system (Promega). Chromosomal DNA was isolated as described previously (31). DNA-modifying enzymes were used according to the manufacturer's recommendations (New England Biolabs).

Construction of disruption mutants. Mutants were constructed by homologous recombination as described elsewhere (45). PCR fragments containing an internal fragment were produced with GoTaq Flexi DNA polymerase (Promega), gel purified (Promega), and cloned into the single NcoI site of the pAP1 suicide vector (31). Design of primers (Table 1) included the addition of sites for the NcoI-compatible PciI restriction endonuclease. To avoid expression of shorter but potentially functional products of these genes, two stop codons (TGATAG) were added in frame at the beginning of forward primers. After transformation of electrocompetent cells of *R. equi*, knockout mutants were selected on apramycin-LB agar plates and subsequently confirmed by colony PCR using internal primers against the apramycin cassette and external gene-specific primers (Table 1). Mutants were assayed for their sensitivity to low doses of 2,2′-dipyridyl as described above. A revertant was obtained by growing *R. equi* mutants in the absence of apramycin. Resulting apramycin-sensitive revertants were tested for growth in the presence of 160 μM 2,2′-dipyridyl. The absence of the disruption plasmid in the genomes of the revertants was determined by PCR.

Production and analysis of iron-chelating compounds. *R. equi* was grown in LMM and LMM-Fe; aliquots (1 ml) were centrifuged at 20,000 × *g* at 4°C for 10 min, followed by filtration of the resulting supernatant through 0.22-μm membrane filters (Millipore). Spectra (300 to 700 nm) of supernatants were obtained in a double-beam Varian spectrophotometer (Varian Inc.). The formation of iron complexes was detected after addition of Fe^{3+} to the supernatants of *R. equi* and mutant strains. Spectra of samples obtained from high-iron conditions of growth were subtracted from those of samples obtained under iron-limited conditions. Catecholate-containing compounds were assayed with Arnow's nitrite-molybdate reagent (2), and 2,3-dihydroxybenzoic acid was used as a standard.

Real-time PCR. Total RNA was isolated from *R. equi* as previously described (29). cDNA was produced by extension of hexameric random primers with Improm II reverse transcriptase, using 100 ng of RNA and following the manufacturer's (Promega) instructions. Real-time PCR to quantify the number of transcripts was performed in a LightCycler (Roche) using the QuantiTect SYBR green PCR kit following the manufacturer's (Qiagen) recommendations. Melting curves were determined at the end of 45 amplification cycles to ensure specificity of the fluorescence signal. Standard curves for known amounts of template DNA were determined in the range of 10^2 to 10^7 molecules.

Virulence assays. The effect of mutation of the *iupS* and *iupU* genes on the virulence of *R. equi* was assayed in the mouse model as previously described (14).

RESULTS

Growth of *R. equi* under low-iron condition. We previously characterized *R. equi* α5, a mutant unable to grow in the presence of low (80 μM) concentrations of the iron chelator 2,2′-dipyridyl due to disruption of a putative siderophore ABC transport system (29). In order to study growth of *R. equi* α5 and the wild type at low iron concentrations in the absence of iron-chelating agents, the medium was treated with Chelex-100. The iron concentration in iron-replete medium (LMM) was determined as 3.71 ± 0.28 μM (n = 4), whereas the iron concentration in LMM-Fe medium was below the assay's detection limit (0.6 μM).

The growth rate of the wild-type strain in LMM-Fe medium was significantly lower than when the strain was grown in iron-replete LMM medium. However, despite the lower growth rate, the final OD of the culture grown in LMM-Fe medium was approximately the same as that obtained in LMM medium (Fig. 1). Growth of *R. equi* α5 was indistinguishable from that of the wild-type strain when grown in iron-replete

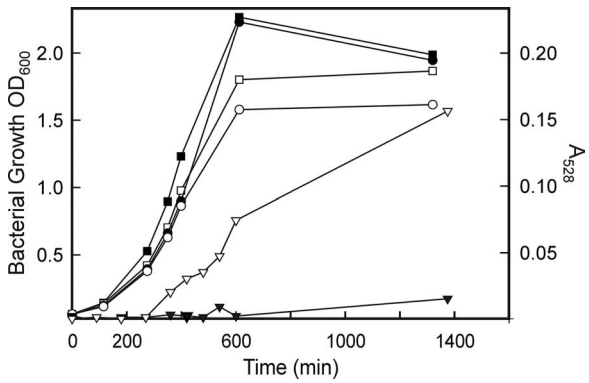

FIG. 1. Growth of *Rhodococcus equi* α5 and wild-type strains in iron-replete (closed symbols) and iron-depleted (open symbols) media. Shown are bacterial growth of *R. equi* α5 (circles) and wild-type *R. equi* (squares) measured as the OD_{600} and absorbance of the medium supernatant at 528 nm in the presence of iron (triangles).

medium; however, both the growth rate and final OD of *R. equi* α5 were lower than those of the wild-type strain when grown in LMM-Fe medium (Fig. 1). These data were consistent with the ABC transport system, absent in the *R. equi* α5 mutant, being involved in the acquisition of iron by *R. equi*.

***R. equi* produces a chromophore during growth at low iron concentrations.** A reddish color developed during growth of *R. equi* α5, but not during growth of the wild-type strain, in iron-replete medium (data not shown). Since *R. equi* α5 carries a transposome insertion in *iupABC*, encoding a siderophore uptake system, we speculated that the red color of the medium supernatant was due to the formation of an Fe^{3+}-siderophore complex, with iron being derived from the Vishniac trace element solution. If so, the absence of a red color in the supernatants of the *R. equi* α5 and wild-type strains following growth in LMM-Fe medium was due to the extremely low concentration of iron. To test this hypothesis, Vishniac trace element solution was added to the supernatants of the *R. equi* α5 and wild-type strains grown in LMM-Fe medium, resulting in the formation of a red color (data not shown). This chromophore was not observed when supernatant of the wild-type strain grown in LMM medium was used or when Vishniac trace element solution lacking $FeSO_4$ was added instead.

Spectroscopic analysis of the culture supernatants of *R. equi* α5 and the wild-type strain following growth in LMM-Fe showed an absorption peak at 341 nm (Fig. 2A). Addition of 36 μM Fe^{3+} to these supernatants resulted in the formation of a second, broad absorption peak with an absorption maximum at 528 nm (Fig. 2A). In contrast, following growth in iron-replete medium neither absorption peak was observed in culture supernatants of the wild-type strain (Fig. 2A), whereas both were present in the culture supernatant of *R. equi* α5 (data not shown). The chromophore(s) started to accumulate during the early exponential growth phase and continued to accumulate in the stationary phase during growth of *R. equi* in LMM-Fe but not in LMM medium (Fig. 1).

The absorption spectra of the supernatants of *R. equi* grown in LMM-Fe medium are similar to that of catecholate-containing siderophores (39). Therefore, the presence of catecholates in culture supernatants of *R. equi* α5 and wild-type strains

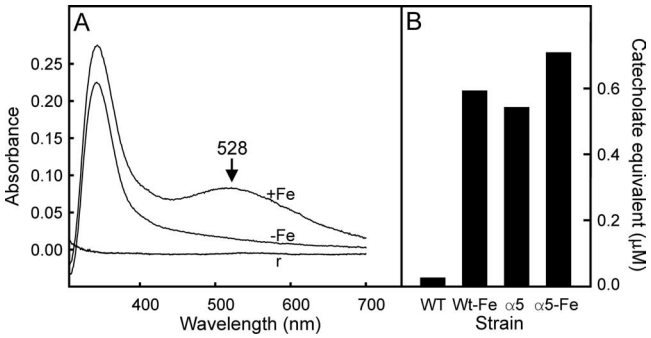

FIG. 2. Supernatants of *R. equi* following growth in iron-replete (LMM) and iron-depleted (LMM-Fe) media. (A) Absorption spectra of the supernatant of *R. equi* following growth in iron-depleted medium. Without addition of $FeSO_4$ to the supernatant, an absorption peak at 341 nm is present (−Fe spectrum); addition of 36 μM $FeSO_4$ to the supernatant gives raise to a second absorption peak at 528 nm (+Fe spectrum, indicated by an arrow). r, absorption spectrum of the supernatant of *R. equi* following growth in iron-replete medium; 36 μM $FeSO_4$ was added to the supernatant. The spectra shown are the result of subtracting the spectrum of the LMM growth medium from that of the LMM-Fe medium. (B) Presence of catecholates in the medium of *R. equi* following growth in LMM or LMM-Fe medium. WT, *R. equi* wild-type strain grown in LMM; WT-Fe, *R. equi* wild-type strain grown in LMM-Fe; α5, *R. equi* α5 grown in LMM; α5-Fe, *R. equi* α5 grown in LMM-Fe.

grown on LMM and LMM-Fe medium was determined. The catecholate content of *R. equi* wild-type supernatants was 18-fold higher following growth in LMM medium than following growth in LMM-Fe medium. In contrast, the *R. equi* α5 strain produced catecholates in both media to the same level as the wild-type strain grown in iron-deplete medium (Fig. 2B).

Identification of putative siderophore biosynthesis genes. The above data strongly suggested that *R. equi* produces a catecholate-containing siderophore during growth in low-iron medium. Catecholate-containing siderophores are generally produced by NRPSs. Analysis of the *R. equi* genome (http://www.sanger.ac.uk/Projects/R_equi/) revealed seven NRPS-encoding gene clusters, two of which were considered most likely to be involved in producing the putative siderophore described above (Fig. 3). The *iupU* gene (nucleotides [nt] 2506417 to 2533221), encoding a protein of 960.3 kDa containing seven NRPS modules, is located 28 kb upstream of *iupABC*, encoding a siderophore uptake system (29). The close proximity to *iupABC* and the linkage with *mbtH*, a gene required for production or secretion of siderophores and frequently clustered with siderophore biosynthesis genes (11, 47), indicated that the *iupU* gene could be involved in siderophore biosynthesis.

The second cluster (nt 851218 to 876561), of 25 kb, contains 10 genes apparently organized into two divergently transcribed operons. A six-cistron operon (nt 855052 to 876561) harbors two large genes encoding proteins of 271.5 and 324.6 kDa with two NRPS modules each (respectively, *iupS* [nt 855052 to 862794] and *iupT* [nt 864015 to 873179]). The remaining four genes are homologous to genes encoding siderophore biosynthetic and transport systems. Upstream and transcribed divergently from this operon is a four-cistron operon (nt 854874 to 850568) encoding homologues of DhbBEAC enzymes required for the production of the catecholate siderophore bacillibactin (26), suggesting that *iupS* and *iupT* may be involved in the biosynthesis of a catecholate siderophore.

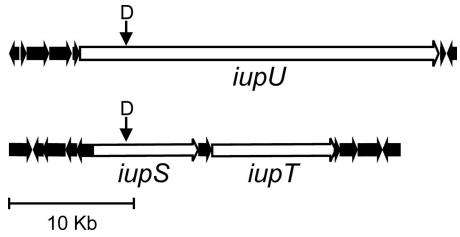

FIG. 3. Genetic context of the *iupU* (located at nt 2506417 to 2533221), *iupS* (located at nt 855052 to 862794), and *iupT* (located at nt 864015 to 873179) genes. The coordinates refer to the genomic sequence of *R. equi*. The position of the gene disruption (D) is indicated by an arrow.

Transcription of *iupS* and *iupT*, but not *iupU*, is regulated by iron. Transcription of genes involved in siderophore biosynthesis, secretion, and uptake is usually regulated by the iron concentration of the growth medium, as was shown previously for *iupABC* in *R. equi* (29). The copy number of the *iupS* and *iupT* transcripts was increased by 3 orders of magnitude during growth in LMM-Fe compared to LMM medium. In contrast, transcription of *iupU* was constitutively high and not regulated by iron; *iupU* transcript levels at low and high iron concentrations were comparable to those of *iupS* and *iupT* during growth at low iron concentrations (Fig. 4).

The *R. equi* SID3 mutant fails to produce a diffusible compound required for growth at low iron concentrations. To determine whether *iupS*, *iupT*, and *iupU* play a role in iron metabolism of *R. equi*, mutants were generated by integrating a small plasmid (pAm1) conferring apramycin resistance and harboring an internal fragment of either *iupS*, *iupT*, or *iupU* into the genome via homologous recombination as described previously (29). The *iupS* and *iupU* mutants were readily obtained and subsequently validated by PCR and Southern hybridization, resulting in, respectively, *R. equi* SID3 and *R. equi* SID1 (data not shown). However, despite repeated attempts, we were unable to generate an *iupT* disruption mutant. The *R equi* SID1 and SID3 strains were unable to grow at relatively

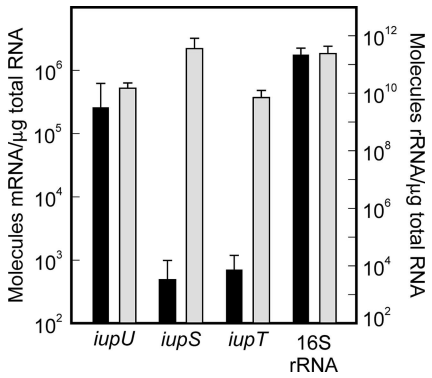

FIG. 4. Absolute quantification of *iupU*, *iupS*, and *iupT* mRNAs and 16S rRNA using RNA isolated from *R. equi* grown under iron-replete (black bars) or iron-depleted (gray bars) conditions. Shown are the averages and standard deviations from two independent experiments in which each sample was analyzed in duplicate.

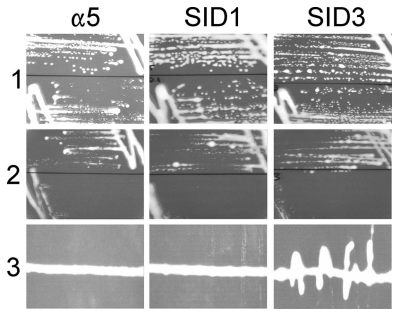

FIG. 5. Growth inhibition by the iron chelator 2,2′-dipyridyl and cross-feeding of the *R. equi* mutants by diffusible compounds secreted by the wild-type strain. Growth of *R. equi* α5, SID1, and SID3 on agar plates containing 0 (row 1) or 160 μM (row 2) 2,2′-dipyridyl is shown. The top half of each panel represents the *R. equi* wild-type strain, whereas the *R. equi* mutant strains are shown in the bottom half of each panel. Row 3, cross-feeding of *R. equi* α5, SID1, and SID3 by the wild-type strain. *R. equi* mutants were zigzag-streaked on LB agar plates containing 160 μM of 2,2′-dipyridyl, a concentration completely inhibiting growth of the mutants. A single streak of wild-type *R. equi* was made across that of the mutant strains. Cross-feeding was recorded after incubation of the plates at 37°C for 3 days.

low concentrations of 2,2′-dipyridyl, a phenotype similar to that of *R. equi* α5 (Fig. 5). This phenotype was not due to an increased sensitivity of the mutant strains to 2,2′-dipyridyl, since addition of $FeCl_3$ allowed growth to resume.

If the inability of *R. equi* SID1 and *R. equi* SID3 to grow under low-iron conditions is due to their inability to produce a diffusible iron-chelating compound, then the wild-type strain which does produce this compound should be able to cross-feed the mutants. To test this possibility, *R. equi* SID1 and SID3, as well as *R. equi* α5 (*iupA* disruption mutant), were streaked in a zigzag pattern on LB agar plates containing 2,2′-dipyridyl at a concentration inhibitory for the mutants but not the wild-type strain. The *R. equi* wild-type strain was subsequently streaked across the mutant strains. The presence of the wild-type strain allowed growth of *R. equi* SID3 at normally inhibitory concentrations of 2,2′-dipyridyl (160 μM), whereas *R. equi* α5 and *R. equi* SID1 failed to grow (Fig. 5).

These data are consistent with *R. equi* SID3 failing to produce a diffusible siderophore. This siderophore could indeed correspond to the chromophore(s) detected in the culture supernatant of the wild-type strain grown under iron-depleted conditions, as the supernatant of *R. equi* SID3 grown in LMM or LMM-Fe did not contain a compound with an absorption maximum at 341 nm and its ferric complex with an absorption maximum at 528 nm, whereas the supernatant of *R. equi* SID1 did. An apramycin-sensitive revertant of *R. equi* SID3 had lost the disruption plasmid from *iupS* and was able to grow in the presence of 160 μM 2,2′-dipyridyl, comparable to the wild type strain.

***iupS* and *iupU* are not required for virulence.** To determine whether either *iupS* or *iupU* is required for virulence of *R. equi*, the virulence of *R. equi* mutants SID1 and SID3 was compared to that of the wild type (and a virulence plasmid-free isogenic derivative as an avirulent control [9]) in a mouse model of systemic infection (9). Although immunocompetent mice will eventually clear *R. equi*, the increase in *R. equi* numbers in liver at 2 to 4 days after injection provides a good indication of *R. equi* virulence in vivo (14). There was no significant difference between the numbers of *R. equi* SID1 and *R. equi* SID3 versus those of the wild type in the liver (6.34 ± 0.17 and 6.38 ± 0.63 versus 6.05 ± 0.67 $\log_{10}$/g, respectively) in mice at 4 days after intravenous injection of the same numbers of bacteria, indicating that these iron acquisition determinants are not critical for within-host growth in vivo, at least in our experimental system.

DISCUSSION

Although it has been reported that *R. equi* requires iron for growth (19), little information is available regarding the mechanisms that this pathogen employs to meet its iron demands in either saprophytic or pathogenic growth conditions. The data presented in this paper provide evidence that the *iupS* gene, encoding an NRPS, is required for the production of a siderophore.

The *iupS* gene is the first gene of what, based on the very small intergenic regions, appears to be a six-cistron 21-kb operon, flanked by divergently transcribed genes. Disruption of *iupS* rendered the resulting mutant unable to grow in the presence of low concentrations of 2,2-dipyridyl and, in addition, dramatically reduced the growth rate in media containing extremely low iron concentrations. This phenotype could be rescued by coculture with the wild-type strain, showing that *R. equi* SID3 is unable to produce a diffusible compound required for growth at low iron concentrations. This conclusion was further supported by the observation that the wild-type strain produces a chromophore during growth in iron-depleted, but not iron-replete, medium. This chromophore is characterized by an absorption maximum at 341 nm and upon the addition of iron a second broad peak with an absorption maximum at 528 nm, leading to the formation of a reddish color. The synthesis of the chromophore and transcription of *iupS* are both regulated by the concentration of iron in the medium, suggesting that these are connected. This connection was demonstrated by the observation that the chromophore is not produced by the *iupS* knockout mutant SID3.

Based on this, we conclude that *iupS* and/or genes located downstream from it in the same six-cistron operon are required for synthesis of the siderophore that is secreted in the medium during growth at low iron concentrations.

The only rhodococcal siderophores that have been characterized to date are heterobactins and rhodobactin which are produced by *Rhodococcus erythropolis* and *Rhodococcus rhodochrous*, respectively. Both siderophores are mixed-ligand siderophores containing hydroxamate and catecholate moieties (6, 10). Spectroscopic analysis of the culture supernatant suggested that the chromophore produced by *R. equi* contains a catecholate moiety. The catecholate moiety of these siderophores, 2,3-dihydroxybenzoate, displays an absorption peak at 315 nm that arises from the $\pi \rightarrow \pi^*$ transition of catechols. Deviation in the wavelength at which maximum absorption occurs has been observed, depending on metal complexation and on the arrangement of hydroxyl groups on the benzene ring of dihydroxybenzoate (21). Upon addition of Fe^{3+}, an absorption peak at 528 nm appeared, which may arise from the ligand-to-metal charge transfer band observed when catecholates bind Fe^{3+}. This absorption peak therefore probably

represents the ferri form of the *R. equi* siderophore. Ferric chrysobactin, the catecholate-containing siderophore of *Erwinia chrysanthemi*, is an Fe^{3+}-bis-catecholate with an absorption maximum at 525 nm at pH 7.4 (32). In addition to the spectroscopic data, accumulation of catecholates in the medium during growth in iron-depleted, but not iron-replete, medium was observed, matching the appearance of the chromophore. Although the presence of other iron-coordinating functionalities cannot be ruled out, these findings strongly suggest that the siderophore produced by *R. equi* includes catecholate moieties.

The *R. equi* α5 mutant carries a transposome insertion in the first gene of the three-cistron *iupABC* operon, encoding a putative siderophore ABC transport system (29). This mutant strain accumulated both the chromophore and catecholates during growth under iron-replete conditions, whereas the wild-type strain did not. The simplest explanation for this phenotype is that the *iupABC*-encoded transport system is responsible for uptake of the siderophore produced by the products of the *iupS* operon. However, this remains to be formally proven.

Expression of the *iupS* and *iupT* genes was coordinately regulated, with transcription levels 3 to 4 orders of magnitude higher in the absence of iron compared to when iron was present. Furthermore, the putative catecholate siderophore was not produced during growth of *R. equi* on medium containing 3.6 μM $FeSO_4$, resembling the production of rhodobactin, which is completely repressed in the presence of 3 μM iron (10). The genome of *R. equi* harbors two iron repressors, Fur and IdeR (http://www.sanger.ac.uk/Projects/R_equi/); the latter has been shown to be functional in *R. equi* (4). If *R. equi* iron regulation is comparable to that in the related *Mycobacterium tuberculosis*, which also has both Fur and IdeR, then IdeR predominantly regulates iron metabolism, including siderophore biosynthesis (35), whereas Fur controls the oxidative stress response (48). The IdeR homologues DtxR and DmdR are responsible for iron-dependent transcriptional regulation of genes required for siderophore biosynthesis in, respectively, *Corynebacterium* (38) and *Streptomyces* (13, 15).

Transcription of the *iupU* gene was not regulated by the concentration of iron in the medium and was at a level comparable to that of fully induced *iupS* and *iupT* during growth under iron-depleted conditions. Transcription of siderophore biosynthesis genes is usually tightly controlled by iron, suggesting that *iupU* is not involved in iron acquisition. However, disruption of *iupU* did prevent growth of *R. equi* at low iron concentrations, i.e., the same phenotype as *iupS* and *iupA* mutations (29). In contrast to *R. equi* SID3, *R. equi* SID1 could not be cross-fed by the wild-type strain, suggesting that the product of IupU either is not secreted or is not diffusible in water. These characteristics are reminiscent of the mycobactin siderophores of mycobacterial species. Thus, in *M. tuberculosis*, for example, extracellular siderophores (carboxymycobactins) donate their iron to the cell wall-bound, nondiffusible mycobactin. Following reduction of ferric to ferrous iron, iron is subsequently transported across the cell membrane (34). Whether the product of IupU fulfills a role similar to that of mycobactin remains to be established.

The ability of *R. equi* SID1 and *R. equi* SID3 mutants to proliferate in the mouse model was not significantly different than that of the wild type, as determined by liver clearance experiments. This strongly suggests that *R. equi* employs additional iron acquisition systems to allow proliferation in the host. The same phenotype was observed previously for *R. equi* α5, which retained wild-type ability to proliferate in macrophages and mice (29). The deployment of multiple, redundant iron uptake systems is not uncommon in pathogenic bacteria. For example, *Burkholderia cenocepacia*, *Bacillus anthracis*, and *Pseudomonas aeruginosa* produce two types of siderophores, yet only one is required for virulence (7, 42, 44). The data presented here therefore identified a catecholate-containing siderophore that is dispensable for within-host growth but required for saprophytic growth under low-iron conditions. Our data imply that *R. equi* may possess additional iron acquisition systems which may be specifically relevant for proliferation during parasitic life.

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